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Isolation of BPgp70, a fibroblast receptor for the envelope antigen of Rauscher murine leukemia virus

(leukemia virus receptor/antibody to virus receptor/gp70 glycoprotein/protein shedding)

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ABSTRACT **A protein that avidly binds gp70, the envelope antigen of Rauscher murine leukemia virus (RMuLV), has been purified from the culture medium used for growth of BALB/c 3T3 mouse cells. Gel filtration chromatography revealed the presence of a single gp70 binding component, BPgp70, of apparent M_r 10,000. BPgp70 was efficiently labeled when BALB/c 3T3 cells were grown in medium containing [^{3}H]leucine, indicating a cellular origin for BPgp70. Metabolically labeled [^{3}H]BPgp70 was not immunoprecipitated by IgG-anti RMuLV-gp70 alone, but was immunoprecipitated when gp70 was added, an indication of BPgp70·gp70 complex formation. The dissociation constant estimated by immunoprecipitation agreed with the apparent K_d for binding of gp70 to BALB/c 3T3 cells. BPgp70 reversibly inhibited specific binding of ^{125}I-labeled RMuLV-gp70 to BALB/c 3T3 cells when it was incubated with the ^{125}I-labeled gp70 first. These data yielded a dissociation constant similar to that calculated from the immunoprecipitation data. ^{125}I-Labeled BPgp70 also bound specifically to cells infected with RMuLV, but not to uninfected cells. Incubation of BALB/c 3T3 cells with the IgG fraction of an antiserum to BPgp70 inhibited the specific binding of ^{125}I-labeled gp70 to these cells, but preimmune IgG did not. Complete inhibition was achieved at a less than 100:1 ratio of IgG anti-BPgp70 to gp70 binding sites.**

Infection of host cells by retroviruses is initiated by specific high-affinity interactions between the viral envelope and the cell surface; this has been revealed by host-range, interference, and neutralization properties within subgroups of avian oncornaviruses (1). Virus strains that lack the envelope glycoprotein neither bind to nor infect normally permissive cells (2, 3).

A major step in the elucidation of the role of viral envelope macromolecules in infection was the purification and characterization of a biologically active 70,000-dalton glycoprotein (gp70) from the envelopes of several murine viruses (4). This glycoprotein is the predominant viral surface antigen and binds specifically to susceptible murine cells, but not to other mammalian cells (5). A binding assay for gp70 was developed with murine 3T3 cells and revealed approximately 5×10^5 sites per cell (5). This binding is highly specific and saturable (5–8). Purified gp70 also binds to isolated membranes, and only one species of receptors has been defined kinetically for a given gp70 species (6, 8). Binding is prevented by antibody prepared against gp70 (5) and also is blocked by cellular production of related type C viruses, presumably because the receptors are occupied by endogenously produced gp70 (5).

Two classes of gp70 receptors have been indicated: those binding ecotropic or xenotropic viruses or gp70 derived from them (6, 7). Although there is compelling evidence that gp70 is responsible for the binding of viral particles to cells and that a specific host cell membrane component is involved in virus recognition (9), the identification of this component has not been described. In this article we report the isolation and preliminary characterization of a gp70 binding protein (BPgp70) for Rauscher murine leukemia virus (RMuLV).

MATERIALS AND METHODS

Cells and RMuLV-gp70. BALB/c 3T3 mouse cells, clone A31 (American Type Culture Collection), were grown in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum (GIBCO). Cells used in binding studies were grown for at least three doublings after passage. JLS-V9 cells, RMuLV-infected JLS-V9 (JLS-V9-RMuLV) cells, and RMuLV grown in chronically infected JLS-V9 cells were provided by the Division of Cancer Cause and Prevention, National Cancer Institute. RMuLV-gp70 was purified as described by Strand and August (4) and radioiodinated by the chloroglycoluril method (10); the specific activities ranged from 10^4 to 10^5 cpm/ng.

Purification of BPgp70. CNBr-activated Sepharose 4B (Pharmacia) was incubated for 2 hr at 24°C and an additional 18 hr at 4°C with 20 μg of RMuLV-gp70 per ml of settled beads in a solution of 100 mM $NaHCO_3$, pH 8.2, and 500 mM NaCl. The reaction yield was nearly 100%. A 1 cm × 30 cm column of gp70-Sepharose 4B equilibrated with 20 mM 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (Bes), pH 7.3, was used to purify BPgp70.

BALB/c 3T3 cells were grown for four doublings to confluence in the minimal essential medium plus 10% fetal calf serum with no medium change. One liter of spent culture fluid used for the growth of 10^9 cells was concentrated to 50 ml and dialyzed against 5 liters of 20 mM Bes, pH 7.3. This material was applied to the affinity column at 24°C, the column was washed with 4 vol of 20 mM Bes, pH 7.3, and protein was eluted with 300 mM NaCl in 20 mM Bes, pH 7.3, at a flow rate of 1 ml/min. Fractions (1 ml) were collected, the fractions containing protein were pooled, and an aliquot was chromatographed on Sephadex G-25 (1 cm × 20 cm), using 20 mM Bes, pH 7.3, as running buffer. Purified BPgp70 was dialyzed against 50 mM Bes, pH 7.3, and stored frozen at −70°C.

Metabolic Labeling of BPgp70. BALB/c 3T3 cells were seeded at a density of 3×10^3 per cm^2 and grown for four generations with no medium change in medium 199 (GIBCO) containing 10% dialyzed fetal calf serum and modified to contain 1 mg of L-leucine and 0.5 mCi (1 Ci = 3.7×10^{10} becquerels) of L-[^{3}H]leucine (Amersham Buchler, Arlington

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Abbreviations: gp70, 70,000-dalton viral coat glycoprotein; BPgp70, binding protein for gp70; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; RMuLV, Rauscher murine leukemia virus; reaction buffer, Dulbecco's modified Eagle's minimal essential medium containing 50 mM Bes (pH 7.3) and 1% bovine serum albumin.

Heights, IL) per 100 ml. BPgp70 was purified from the culture supernatant fluid used for growth of 1.2×10^7 metabolically labeled cells. The specific activity of ^{3}H-labeled cellular protein was determined by mixing 1.5×10^5 cells with 10 mg of albumin in 1 ml of water, precipitating with 4 ml of 95% (vol/vol) ethanol (1 hr at 0°C) and washing the precipitate twice with 2 ml of ethanol, twice with 2 ml of methanol, and once with 2 ml of diethyl ether prior to scintillation counting. Protein was determined by micro adaptations of published methods (11, 12).

Antisera. IgG fractions were produced from antisera raised in rabbits and from preimmune sera (13).

Binding of ^{125}I-Labeled gp70 to 3T3 Cells. Binding of ^{125}I-labeled gp70 (^{125}I-gp70) to confluent BALB/c 3T3 cell monolayers was assayed in duplicate in 16-mm wells (1–1.5 × 10^5 cells per well). Cells were incubated for 60 min at 24°C with 100 ng of ^{125}I-gp70 in 300 μl of reaction buffer (Dulbecco's modified Eagle's minimal essential medium containing 50 mM Bes at pH 7.3 and 1% bovine serum albumin) and washed three times with reaction buffer. The contents of the well were suspended in 500 μl of 50 mM Tris·HCl, pH 6.8, containing 2% sodium dodecyl sulfate and the radioactivity was determined by γ spectrometry. Specific binding was the difference between binding of ^{125}I-gp70 in the absence and presence of 1 μg of unlabeled gp70.

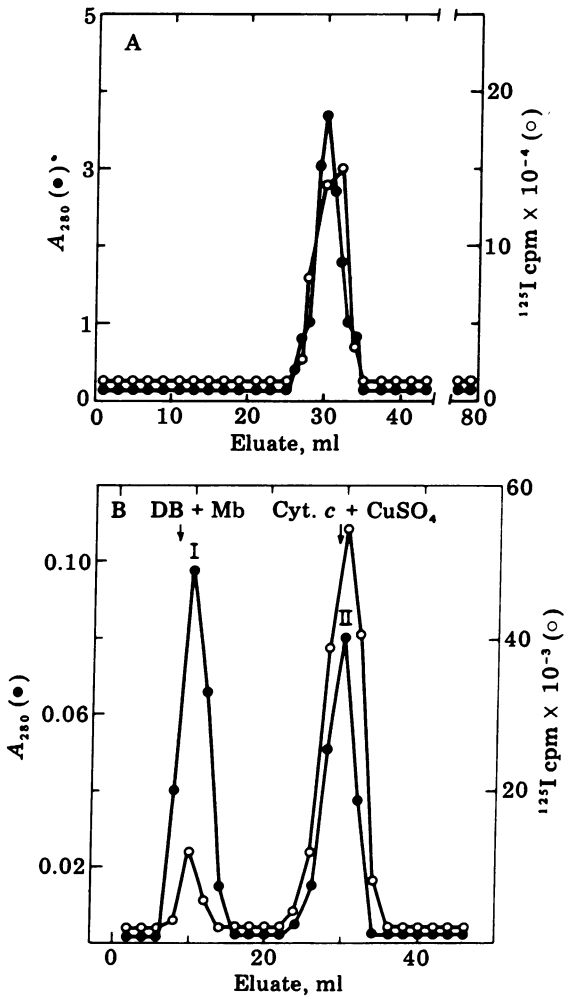


FIG. 1. Purification of BPgp70. (*A*) Elution from gp70-Sepharose. Proteins from 1 liter of spent culture medium from growth of 10^9 BALB/c 3T3 cells were radioiodinated with 5 mCi of Na^{125}I (10) and applied to a RMuLV-gp70-Sepharose column. The adhering proteins were eluted as described in *Materials and Methods*. (*B*) Gel filtration of proteins eluted from gp70-Sepharose. Fractions (1 ml) were tested for A_{280} and ^{125}I radioactivity. The column was characterized with dextran blue (DB), myoglobin, cytochrome *c*, and $CuSO_4$. Over 90% of the applied radioactivity and 280-nm-absorbing material was recovered.

RESULTS

Isolation of BPgp70. Proteins from the medium used for growth of BALB/c 3T3 cells were radioiodinated and applied to gp70-Sepharose, and adsorbed proteins were eluted with high ionic strength solution (Fig. 1*A*). This procedure was suggested by the report that high ionic strength dissociates specifically bound gp70 from cells (6). Approximately 12 A_{280} units of protein (4.3 mg) was eluted. A 0.5-ml sample (215 μg, 0.6 A_{280} unit) was purified further on Sephadex G-25 (Fig. 1*B*). Peak I consisted mainly of albumin (175 μg). Peak II material was eluted within the inclusion volume and was not immunoprecipitated by antisera to either whole fetal calf serum or gp70. It had a high A_{280} per mg of protein (11–12). The peak II fractions rich in 280-nm-absorbing material and radioactivity were pooled, yielding 35 μg of protein (0.36 A_{280} unit). The culture supernatant fluid used for growth of 10^9 cells yielded 700 μg of BPgp70.

Molecular Weight of BPgp70. Sodium dodecyl sulfate gel electrophoretic analysis of peak II protein revealed a single major component of apparent M_r 10,000 (Fig. 2). Isoelectric focusing also revealed the presence of a single major component. All efforts to demonstrate BPgp70 after fixation, staining, and autoradiography were unsuccessful.

Metabolic Labeling of BPgp70 with [^{3}H]Leucine. BPgp70 was purified from the culture supernatant fluid of BALB/c 3T3 cells grown for four generations in medium containing [^{3}H]-leucine. The specific activities of total cellular protein and isolated BPgp70 were similar (Table 1), showing that BPgp70 is of cellular origin.

Immunoprecipitation of [^{3}H]BPgp70. Table 2 describes the IgG anti-gp70-mediated immunoprecipitation of metabolically

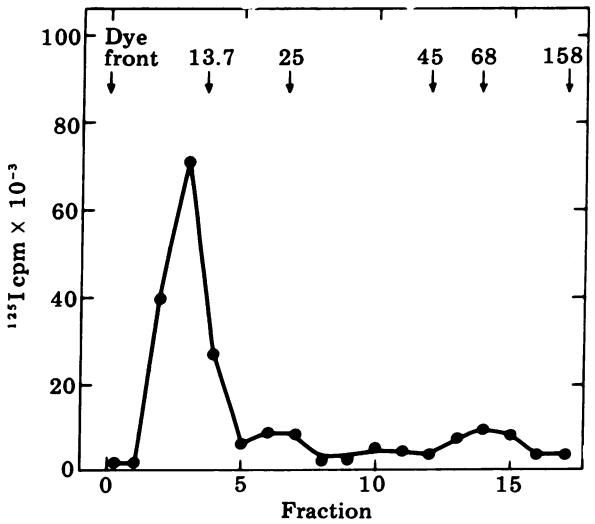


FIG. 2. Gel electrophoresis of ^{125}I-BPgp70 eluted from Sephadex G-25. A 5-μg sample of ^{125}I-BPgp70 was electrophoresed for 8 hr at 25 mA (14), and the gel was cut into 0.7-cm slices for determination of ^{125}I radioactivity. The recovery of radioactivity was 95%. The molecular weight standards (ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and aldolase; $M_r \times 10^{-3}$ is indicated) and BPgp70 were reduced by boiling in reservoir buffer containing 5% (vol/vol) 2-mercaptoethanol.

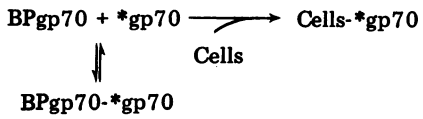
Table 1. Metabolic labeling of BPgp70 with [^{3}H]leucine

Fraction	Specific activity, cpm/mg protein
Total cellular protein	1.3×10^6
BPgp70	1.1×10^6

BALB/c 3T3 cells were grown in medium containing [^{3}H]leucine for four cell doublings. The cells were processed for determination of specific radioactivity in bulk protein and BPgp70 was isolated from the culture supernatant fluid.

labeled [^{3}H]BPgp70 complexed with gp70. A preparation of formaldehyde-fixed *S. aureus* was used to bind IgG and pellet the complexed material (15). Immunoprecipitation of BPgp70 via IgG anti-gp70 required gp70, indicating no crossreactivity, and an unspecific IgG preparation did not substitute for IgG anti-gp70. The data in Table 2 yield a dissociation constant, K_d, of 5 nM for the gp70·BPgp70 complex.

Characterization of the Interaction of BPgp70 with gp70. Incubation of a gp70 binding protein with radioiodinated gp70 should decrease the concentration of "free" gp70 and thus reduce its binding to receptors on cells.



The conditions for detection of this inhibition can be optimized by holding the gp70 concentration within the "linear" range of the binding curve. In the assay system used, gp70 occupied about 10% of the available gp70 receptors. Incubation of BPgp70 with ^{125}I-gp70 inhibited binding of ^{125}I-gp70 to these cells (Fig. 3*A*). The amount of gp70·BPgp70 complex formed was estimated by multiplying the fractional loss of gp70 binding by the total gp70 present. This yields the curve in Fig. 3*B*, which displays typical saturation kinetics and yields a K_d of 30 nM when treated according to Scatchard (Fig. 3*C*) (16). This treatment underestimates the affinity of BPgp70 for gp70, because the complex dissociates during the period of gp70 binding to cells.

The experiment in Fig. 3*D* tests the reversibility of gp70–BPgp70 association. The points at 1 hr of incubation are equivalent to the conditions in Fig. 3*A*. Incubation for an additional 2 hr led to essentially complete restoration of binding activity. This may be explained by the observation that specific

Table 2. Immunoprecipitation of metabolically labeled [^{3}H]BPgp70

System	[^{3}H]BPgp70 immunoprecipitated, pmol
Complete	17
Minus gp70	1
Substitute preimmune IgG for anti-gp70 IgG	3

Twenty picomoles of [^{3}H]BPgp70 and 43 pmol of gp70 were incubated for 60 min at 24°C in 1.0 ml of reaction buffer. One nanomole of the IgG indicated was then added in 100 μl of reaction buffer for a 10-min incubation at 24°C; this was followed by the addition of 200 μl of a 25% wt/vol suspension of *Staphylococcus aureus* in reaction buffer (14). After a 10-min incubation at 0°C, the samples were filtered over Whatman GF/c filters, and the filters were washed with ice-cold reaction buffer in preparation for determination of radioactivity. All values are the mean of two independent determinations.

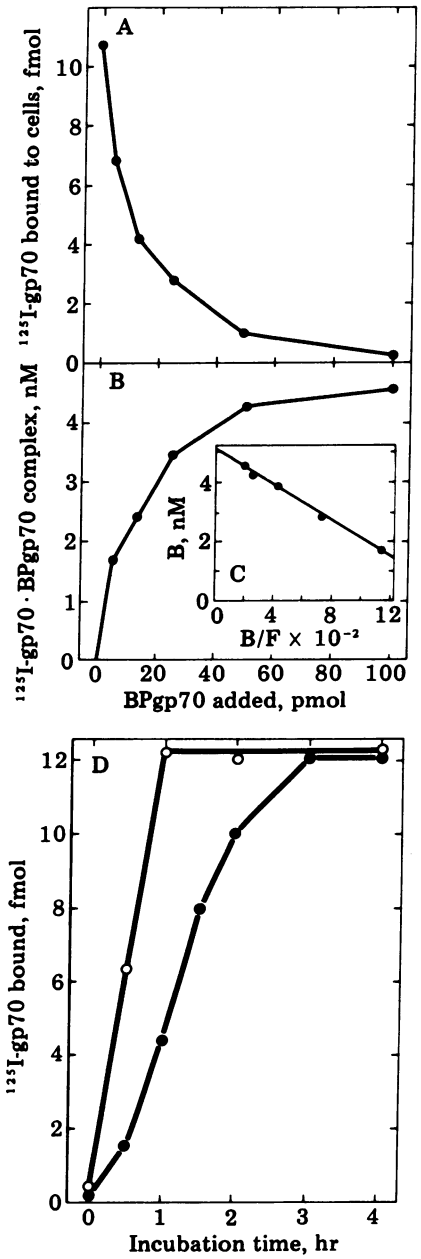


FIG. 3. Interaction of BPgp70 with ^{125}I-gp70. (*A*) Inhibition of ^{125}I-gp70 specific binding to BALB/c 3T3 cells by BPgp70. The indicated amounts of BPgp70 were incubated in 300 μl of reaction buffer for 60 min at 24°C with 100 ng of ^{125}I-gp70. The mixture was then added to 10^5 BALB/c 3T3 cells, and binding of gp70 was determined after an additional 60-min incubation at 24°C. The data plotted are specific binding, which was 90% of total binding. (*B*) Concentration of ^{125}I-gp70·BPgp70 complex formed by incubating ^{125}I-gp70 with BPgp70. The data in *A* were treated as described in *Results* to estimate the gp70·BPgp70 complex concentrations. This assumes that a 1:1 complex of gp70 and BPgp70 is formed and that this complex does not bind to the surface receptors on cells. (*C*) Estimation of the K_d of the ^{125}I-gp70·BPgp70 complex. The data in *B* were treated according to Scatchard (16), yielding a K_d of 30 nM. B, bound ^{125}I-gp70; F, free ^{125}I-gp70. (*D*) Time-dependent dissociation of ^{125}I-gp70 from the ^{125}I-gp70·BPgp70 complex. A 100-ng quantity of ^{125}I-gp70 was incubated with 15 pmol of BPgp70 in 300 μl of reaction buffer for 60 min at 24°C. The mixture was then added to 10^5 BALB/c 3T3 cells and incubated at 24°C. A second set of samples was given the same treatment except that BPgp70 was omitted. The cells were processed for determination of gp70 specific binding. ●, 300 μl of reaction buffer containing 100 ng of ^{125}I-gp70 and 15 pmol of BPgp70 were incubated for 60 min at 24°C prior to addition to cells; ○, 300 μl of reaction buffer containing 100 ng of ^{125}I-gp70 but no BPgp70.

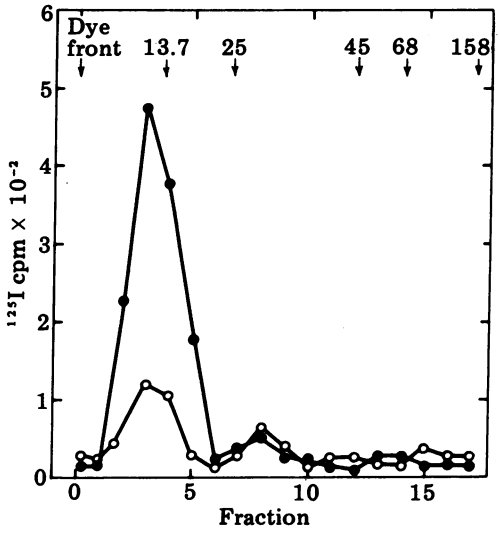


FIG. 4. Gel electrophoresis of ^{125}I-BPgp70 specifically bound to RMuLV-infected JLS-V9 cells. Cultures of 10^5 RMuLV-infected JLS-V9 cells were incubated for 60 min at 24°C with 150 pmol of ^{125}I-BPgp70 in the presence (○) or the absence (●) of 50 μg of unlabeled BPgp70. The cells were then washed twice with reaction buffer and processed for gel electrophoresis as described for Fig. 2. M_r markers are as in Fig. 2. BPgp70 was radioiodinated by the chloroglyoluril procedure (10); the specific activity was 10^5 cpm/μg.

binding of gp70 to BALB/c 3T3 cells is essentially irreversible (8, 17), a finding also reported for NIH 3T3 cells (6). The data in Fig. 3*D* also show that BPgp70 does not inhibit gp70 binding by irreversibly inactivating gp70.

Binding of ^{125}I-BPgp70 to RMuLV-Infected Cells. When JLS-V9-RMuLV cells were incubated with ^{125}I-BPgp70 and the bound radioactivity was analyzed by gel electrophoresis, the ^{125}I radioactivity was located in the M_r 10,000 region (Fig. 4). The detergent extract prepared from JLS-V9-RMuLV cells incubated with a 50-fold excess of unlabeled BPgp70 in addition to ^{125}I-BPgp70 revealed a band that, though identical in location, contained 80% less radioactivity. This indicates that the isolated 10,000-dalton protein is the component that binds

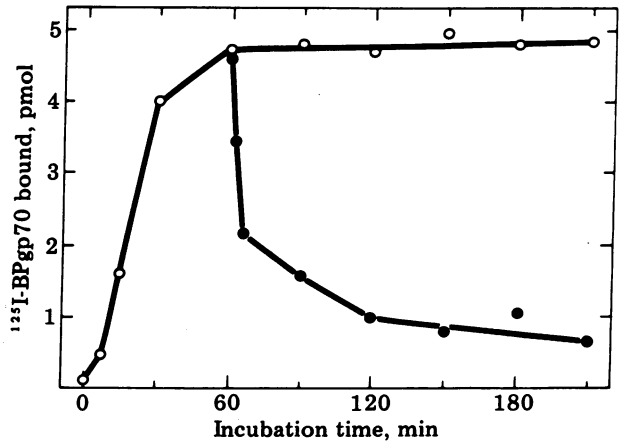


FIG. 5. Time course for binding of ^{125}I-BPgp70 to JLS-V9-RMuLV cells. Samples (10^5 cells) of JLS-V9-RMuLV cells were incubated at 24°C with 150 pmol of ^{125}I-BPgp70 in 300 μl of reaction buffer (○). The cells of an identical set of samples were washed after binding equilibrium was achieved (60 min), and 300 μl of fresh buffer was added to each sample prior to further incubation at 24°C to allow for dissociation of ^{125}I-BPgp70 (●).

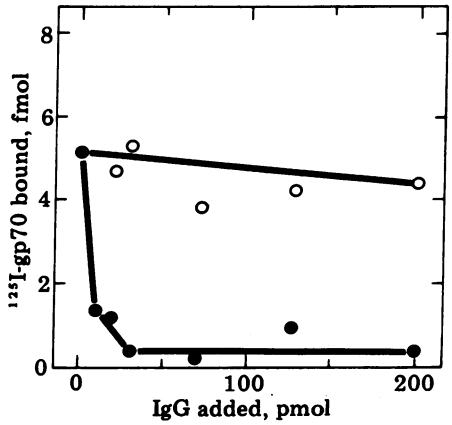


FIG. 6. Inhibition of specific binding of ^{125}I-gp70 to BALB/c 3T3 cells by IgG anti-BPgp70. Cells adhering to 16-mm test wells (approximately 10^5) were incubated for 30 min at 0°C with 300 μl of reaction buffer containing rabbit IgG to occupy the Fc receptors. The cells were then treated with the indicated amounts of IgG anti-BPgp70 (●) or with preimmune IgG (○) in 300 μl of reaction buffer for 30 min at 0°C, washed three times with 1 ml of reaction buffer, and incubated with 100 ng of ^{125}I-gp70 in 300 μl of buffer for 60 min at 24°C. At this point the cells were processed for determination of specific binding, which was approximately 90% of total binding.

specifically to gp70. We have also studied binding of ^{125}I-BPgp70 to BALB/c 3T3 cells and to BALB/c 3T3 cells incubated previously with gp70. Specific binding of BPgp70 was observed only when exogenous gp70 was added (17).

Specific ^{125}I-BPgp70 binding to JLS-V9-RMuLV cells (Fig. 5) reached equilibrium after 60 min. When free ^{125}I-BPgp70 was removed from the incubation medium after equilibrium was achieved, the bound ^{125}I-BPgp70 dissociated rapidly. Over 50% of the bound material was released within 10 min. Specific binding of BPgp70 to JLS-V9-RMuLV cells was saturable with a K_d of approximately 100 nM (data not shown).

Antiserum to BPgp70. Antiserum to peak II fraction BPgp70 was tested for its ability to inhibit gp70 binding to cells when incubated with cells prior to addition of gp70. Immune-IgG to BALB/c 3T3 cell BPgp70 did not immunoprecipitate RMuLV-gp70. Complete inhibition of gp70 binding occurred at a IgG-to-BPgp70 ratio of about 100:1. Fifty percent inhibition was achieved at a ratio of 30:1 (Fig. 6). These data are consistent with the proposition that BPgp70 is a physiological cell surface receptor for gp70 on BALB/c 3T3 cells.

DISCUSSION

Early work by Steck and Rubin (18, 19) pointed to the existence of specific interactions between type C viruses and their host cells. There is compelling evidence for binding of the murine type C viral antigen gp70 to a single high-affinity recognition site on murine cells or isolated cell membranes (5, 6, 8). Receptors for gp70 are likely to play an essential role in the determination of host range and pathogenicity of murine leukemia and sarcoma viruses.

We have shown that BALB/c 3T3 cells shed a 10,000-dalton component with properties expected of a gp70 receptor, and have termed this component a binding protein for gp70 (BPgp70). Shedding of cell surface antigens is a common process and occurs in a variety of other systems—e.g., alloantigen receptors from B and T cells (20) and HLA/H-2 products from lymphocytes (21). BPgp70 is a major secretory product of BALB/c 3T3 cells, and shed BPgp70 was purified in yield sufficient for initial characterization. It has several biochemical properties worth noting. It cannot be fixed in polyacrylamide

gels and has a high A_{280}/mg of protein. These properties have also been described for retinal ligatin, a 10,000-dalton extracellular protein (22).

The apparent ability of gp70 to form complexes by self-association (23) raised concern that BPgp70 isolated by affinity chromatography might be derived from the gp70 coupled to Sepharose. This was excluded by the following properties of BPgp70: (*i*) It is labeled metabolically (Table 1). (*ii*) It binds specifically to RMuLV-infected cells, whereas gp70 does not (Figs. 4 and 5). (*iii*) It is not directly immunoprecipitated by antisera to gp70 (Table 2). The metabolic labeling and immunoprecipitation data also show that BPgp70 is not derived from a serum component in the culture medium.

There are two independent lines of evidence that BPgp70 functions as a RMuLV-gp70 receptor. First, BPgp70 binds avidly to gp70; this was demonstrated directly through binding studies with cells expressing gp70 (Figs. 4 and 5) (17) and by immunoprecipitation of a BPgp70·gp70 complex by an antiserum to gp70 (Table 2). BPgp70·gp70 complex formation was also demonstrated by the ability of BPgp70 to inhibit binding of gp70 to BALB/c 3T3 cells (Fig. 3). Second, exposure of BALB/c 3T3 cells to the IgG fraction of an antiserum raised against BPgp70 specifically and completely inhibited binding of gp70 to these cells (Fig. 6).

The interactions of gp70 with cells or BPgp70 are complex. Experiments based on IgG anti-gp70-mediated immunoprecipitation of the gp70·BPgp70 complex yielded a K_d of approximately 5 nM (Table 2), a value within experimental error of the apparent K_d for binding of gp70 to BALB/c 3T3 cells. A similar apparent K_d value has been reported for interaction of RMuLV-gp70 with membranes isolated from BALB/c 3T3 cells (8), but the apparent K_d with NIH 3T3 cells is 1/10th of this (6). The K_d determined indirectly by means of the ability of BPgp70 to block gp70 binding to cells was higher (30 nM), but this may be attributed to dissociation of the gp70·BPgp70 complex (Fig. 3*D*) and the failure of the complex of gp70 with cells to dissociate readily (6, 8, 17). The irreversible nature of the complex of gp70 with cells and the variation in apparent K_d values reported for cell·RMuLV-gp70 complexes suggest complicated binding interactions between gp70 and its cellular receptors. Similar complexities are revealed in our studies on binding of BPgp70 to RMuLV-infected cells, in which the apparent K_d value for the association of cellular gp70 and BPgp70 was 100-fold higher than that estimated for the interaction of free gp70 and BPgp70. These observations show that cell surface organization may greatly affect the strength of gp70–BPgp70 interactions.

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